ORIGINAL ARTICLE

Optimization of bacterial cellulose production using Plackett-Burman and response surface methodology

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ABSTRACT

Background: *Cellulose is the most widely used natural polymer and can be synthesized by both plants and bacteria. Bacterial cellulose (BC) is a natural biopolymer synthesized by some bacteria, among BC producing bacteria, Komagataeibacter strains have attracted more attention among researchers due to their ability to synthesis BC with high yields.* ***Objective:*** *This study was evaluating the BC production by Komagataeibacter rhaeticus N1 MW32270. Optimization of BC production by statistical optimization method was studied.* ***Methodology:*** *Plackett-Burman (PB) Design was used for screening eight tested parameters, then Response surface methodology (RSM) was applied for optimization the significant factors.* ***Results:*** *PB Design revealed that among the eight tested parameters ethanol concentration and incubation time were the most significant factors affecting BC production. RSM was applied for optimization of BC production. The fitted value of ethanol (1.0152%) and incubation time (10 days) were applied; BC production was compared with the predicted value obtained from the optimization plot. According to the achieved results, the BC production by K. rhaeticus N1 MW32270 was 9.2± 1.1g/l in the optimized medium, which agreed well with the predicted values 9.1 g/l, indicating that the model was fitted. Also BC production by K. rhaeticus N1 MW32270 in optimized medium was then compared with BC production in the standard Hestrin and Schramm (HS) medium. Our results showed that BC production increased by 115% when compared with BC production in the standard HS medium.* ***Conclusion:*** *Enhancement of BC production by K. rhaeticus N1 MW32270 from (4.3± 0.5 g/l) to (9.2± 1.1 g/l) was achieved by using PB and RSM.*

INTRODUCTION

Bacterial cellulose (BC) also known as microbial cellulose (MC) or biocellulose is an organic compound produced as an exopolysaccharide by some bacteria[1, 2].

BC is chemically equivalent to plant cellulose; it represents the purest form of cellulose[3, 4]. BC is free from lignin or hemicelluloses**,** thus possesses high degree of purity[5]**.** The rate of BC synthesis in bacteria is approximately 40 times faster than that of plant cellulose[6]**.** The high aspect ratio of BC fibers with a diameter of (20-100nm), provides BC with a very high surface area which results in a high hydrophilic nature and high water holding capacity (up to 200 times of its dry mass) [7]**.** BC produced in static culture is characterized by a three-dimensional (3D) structure consisting of ultrafine nanofibrils (7-8nm), high degree of crystallinity (84-89%), high mechanical properties superior to other celluloses, one hundred times thinner than most plant cellulose and 100 times smaller than those of plant-based fibers [8-10]**.** These properties make BC a preferred choice than plant cellulose for different applications such as carrier in drug delivery systems, 3D scaffolds for tissue engineering, enzyme immobilization, artificial skin burns, wound dressing materials, tissue regeneration, vascular grafts and biosensors[11, 12]**.** BC also has applications in other technological fields as electronic papers, membranes for audio devices, coating paper, and nanocomposites, as well as other uses[13]**.** An economically feasible synthesis is one of the main aims of BC researches; therefore, optimization of medium component for BC synthesis plays an important role in the production process. Statistically based experimental designs have proved to be more efficient than classical methods. Classical methods by changing one factor per time and fixing the other factors have many disadvantages, which are time-consuming, complicated, and do not consider the interactions between different factors under study. On the other hand, statistical experimental designs are

simple, less time consuming and help in understanding the interaction between different important parameters therefore, in recent year's numbers of statistical designs such as PBD and RSM were applied[14-17]. The current study aims to optimize BC production by using PBD and RSM. This is conducted in two steps. The first one, called the screening phase, evaluated the influence of different factors on BC production through PBD. The second step is the optimization phase, which was achieved by using RSM.

METHODOLOGY

Production and purification of BC:

Cellulose producing *Komagataeibacter rhaeticus N1 MW322708* (https://www.ncbi.nlm.nih.gov/nuccore/MW322708) was used in this work.

K. rhaeticus N1 MW322708 was inoculated into a conical flasks that contains the experimental design values. BC was purified by soaking it in NaOH solution (0.1 M) at 85°C for 2 h, and then rinsed several times with deionized water to achieve a neutral pH; finally BC sheets were dried at 85°C for 1hour.

Screening of different variables for BC production by Plackett–Burman design (PBD):

The PBD is a mathematical modeling for identifying the critical factors that mainly affect the response[18]. In this experiment, PBD was used to estimate the significance of many variables towards BC production. Eight individual variables (Temperature, pH, glucose concentration (%), yeast extract concentration (%), peptone concentration (%), ethanol concentration (%), incubation time (day) and media volume (%) were used. Each independent variable was set at two levels: low and high level (table 1). The Minitab software (V18, Minitab Inc., State College, PA, USA) was used. The BC production was calculated as the dry weight of each run.

Table 1: Coded and actual values of the low and high experimental domain used in the optimization of BC production by *K. rhaeticus N1 MW32270*

Variable	Code	Values of coded levels (mg L^{-1})	
		Low (–1)	High (+1)
Temperature	A	25	35
pH	B	5	7
Glucose concentration (%)	C	2	4
Yeast concentration (%)	D	0.3	0.8
Peptone concentration (%)	E	0.3	0.8
Ethanol concentration (%)	F	0	1.5
Incubation time (day)	G	5	10
Media volume (%)	H	20	40

Response surface methodology (RSM) for maximization of BC production

Depending on the results of PBD, factors that showing a significant effect on BC production were selected for further investigation to determine the optimal settings to maximize the BC production using the RSM based on the Central Composite design (CCD). The significant variables were: ethanol and incubation time. For this procedure, 13 experiments were designed for each ethanol and incubation time. The response function (BC production) was optimized by a second-order model. The predicted value of optimum BC production and culture conditions were obtained. To verify the model of the response surface, the experimental value, obtained under the optimum condition, was compared with the predicted value of the BC production.

Validation of BC production of *K. rhaeticus*

To confirm statistical results, BC production yield of K. rhaeticus *N1 MW32270* in optimized medium (fig. 1) was compared with the predicted yield value of BC obtained from the optimization plot. Also BC production yield in optimized medium was compared with BC production yield in standard HS medium (% w/v): 2% glucose, 0.5% peptone– 0.5% yeast extract – 0.5, Na_2HPO_4 – 0.27, and citric acid – 0.15% citric acid[19].

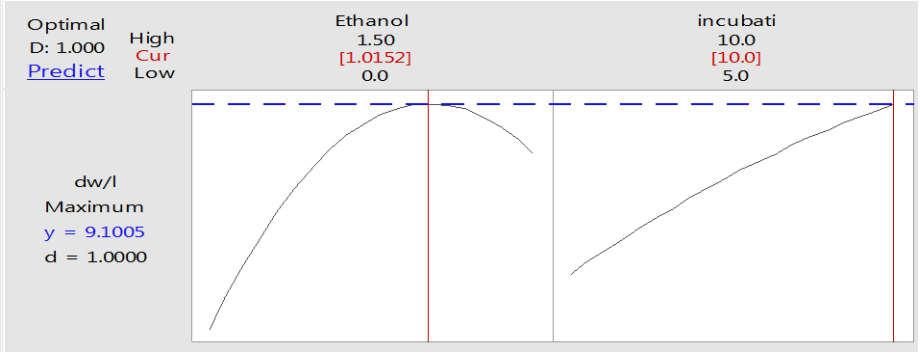

Fig. 1: The optimization plot displays the fitted values for the predictor settings to maximize BC production by *K. rhaeticus N1 MW32270* to 9.1005 g/l

RESULTS

Screening of the independent variables:

The PBD with two levels of 12 runs was used to analyze the effect of 8 individual variables (temperature, pH, glucose concentration, yeast extract concentration, peptone concentration, ethanol concentration, incubation time, and media volume) on BC production by *K. rhaeticus N1 MW32270*

The experimental and predicted data of BC production are illustrated in table 2. The higher yield of BC (7.5 g/l) was obtained at run seven (temperature 25 C°, pH 7, glucose concentration 4%, yeast extract concentration 0.8%, peptone concentration 0.3%, ethanol concentration 1.5%, incubation time 10 days, and media volume 20%). While the lowest yield of BC 1.1 g/l was obtained at run twelve (temperature 25 C°, pH 5, glucose concentration 2%, yeast extract concentration 0.3%, peptone concentration 0.3%, ethanol concentration 0.0%, incubation time 5 days, and media volume 20%).

Table 2: The experimental design, predicted and observed yields of BC production (g/l) by *K. rhaeticus* N1 MW32270 in PBD

Run order	Variables								BC production (g /l)	
	A	B	C	D	E	F	G	H	Experimental value	Predicted value
1	35	5	4	0.3	0.3	0.0	10	40	3.1	3.23333
2	35	7	2	0.8	0.3	0.0	5	40	1.3	0.93333
3	25	7	4	0.3	0.8	0.0	5	20	1.5	1.36667
4	35	5	4	0.8	0.3	1.5	5	20	3.9	4.00000
5	35	7	2	0.8	0.8	0.0	10	20	3.3	3.66667
6	35	7	4	0.3	0.8	1.5	5	40	3.7	3.83333
7	25	7	4	0.8	0.3	1.5	10	20	7.5	7.40000
8	25	5	4	0.8	0.8	0.0	10	40	3.6	3.46667
9	25	5	2	0.8	0.8	1.5	5	40	3.8	3.93333
10	35	5	2	0.3	0.8	1.5	10	20	6.8	6.43333
11	25	7	2	0.3	0.3	1.5	10	40	7.0	7.10000
12	25	5	2	0.3	0.3	0.0	5	20	1.1	1.23333

The results of variance analysis and the determination of parameters are listed in table 3. BC production was negatively correlated with increasing of temperature, glucose concentration, peptone concentration and media volume, whereas increasing the other factors demonstrated a positive effect on BC production.

By applying regression analysis on the results above of both models of species, the equations for BC production were as follows:

The regression equation for BC production by *K. rhaeticus* N1 MW32270

Yield g/l = -0.90 - 0.0400 Temperature + 0.167 pH - 0.000 Glucose concentration(%)
+ 0.067 Yeast concentration(%)- 0.400 peptone concentration(%)
+ 2.089 Ethanol concentration(%)+ 0.5333 Incubation time(day)
- 0.0133 media volume(%)

This regression equation has coefficient (R2) of 98%, indicating that the data in PBD could be explained by the model. Also, the values of, R^2_{adj} and R^2_{pred} *were* 96.2 % and 83.5%, respectively, which revealed that the used models explain very well the obtained data.

Table 3: Statistical analysis of the PBD of BC production (g /l) by *K. rhaeticus* N1 MW32270

Source	DF	Adjust Sum of squares	Adjust Mean square	F-Value	Coefficient	*P*-Value
Model	8	51.9367	6.4921	36.07	3.883	0.007
A	1	0.48	0.48	2.67	-0.200	0.201
B	1	0.3333	0.3333	1.85	0.167	0.267
C	1	0	0	0	-0.000	1
D	1	0.0033	0.0033	0.02	0.017	0.9
E	1	0.12	0.12	0.67	-0.100	0.474
F	1	29.4533	29.4533	163.63	1.567	0.001
G	1	21.3333	21.3333	118.52	1.333	0.002
H	1	0.2133	0.2133	1.19	-0.133	0.356
Error	3	0.54	0.18			
Total	11	52.4767				

R^2 = 98 %; R^2_{adj} = 96.2 %; R^2_{pred} =83.5%

From the Pareto chart (Figure 2) of standardized effects, it was observed that ethanol concentration and incubation time have significant effects on BC production when compared to the other tested factors (temperature, pH, glucose concentration, yeast extract concentration, peptone concentration, and media volume).

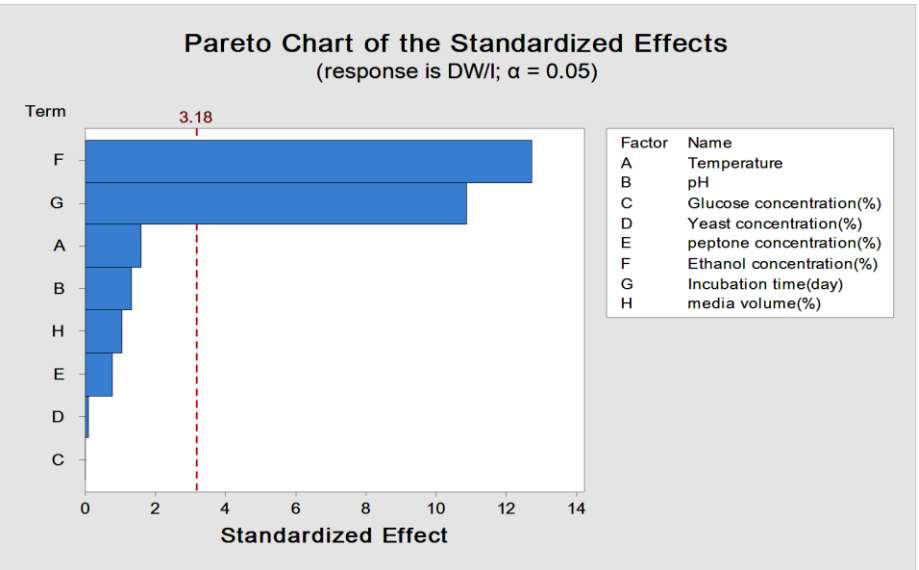

Fig. 2: Pareto Chart showing the significant effect of interacted variables on BC production (g /l) by *K. rhaeticus N1 MW32270*

Identifying the best culture conditions for BC production using Central Composite Design (CCD)

The significant variables impacting BC production of *K. rhaeticus* were chosen to make further optimization by the CCD. Based on the results of the previous models, the CCD was used to further confirm the optimum level of ethanol concentration and incubation time for *K. rhaeticus*, to maximize BC production. In this experiment, four cube points, five center points, and four axial points were required, resulting in a thirteen runs. The values of other parameters were (temperature 25 C°, pH 7, glucose concentration 2%, yeast extract concentration 0.8%, peptone concentration 0.3% and media volume 20%).

Table (4) presents the experimental and predicted values of BC production by *K. rhaeticus N1 MW32270* Among the thirteen runs, run eight (ethanol concentration (0.75%) and incubation time (10 days) offered the highest BC production (8.8 g/l) (fig 3), while run number eleven, ethanol concentration and incubation time were 0% and five days respectively, provided the lowest BC production (1.8 g/l).

Table 4: The experimental design, predicted and observed yields of BC production (g/l) by *K. rhaeticus* N1 MW32270 in CCD

Run order	Point Type[$]	BC production (g/l)			
		Ethanol (%)	Inc. time(days)	Experimental value	Predicted value
1	0	0.75	7.5	7.5	7.355172414
2	-1	1.50	7.5	6	6.228735632
3	0	0.75	7.5	7.8	7.355172414
4	1	1.50	5.0	4.1	3.860632184
5	0	0.75	7.5	7.3	7.355172414
6	1	1.50	10.0	8	8.010632184
7	-1	0.75	5.0	4.9	5.362068966
8	-1	0.75	10.0	8.8	8.762068966
9	0	0.75	7.5	7.3	7.355172414
10	1	0.00	10.0	4.2	4.227298851
11	1	0.00	5.0	1.8	1.577298851
12	0	0.75	7.5	7.3	7.355172414
13	-1	0.00	7.5	3	3.195402299

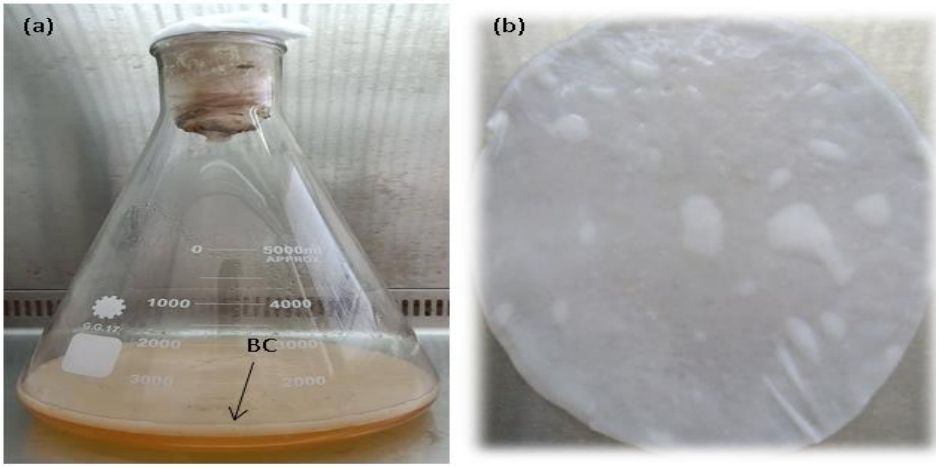

Fig. 3: BC produced by *K. rhaeticus N1 MW32270* grown on optimized media, the figure shows that BC was formed on the top of the optimized medium (a), and BC after purification (b)

To evaluate the efficiency of the model, multiple regression analyses on the data were applied. The results in table 5 represent mainly the individual effects of all variable and their squared and interactions on BC production by *K. rhaeticus* N1 MW32270. The ‘‘Lack of Fit F-value” of 3.12 means the Lack of Fit is insignificant. The insignificance of lack of Fit value is a good indication of the model.

Based on the regression coefficient of the model, there was an interaction between ethanol concentration and incubation time was noted (P-value = 0.042).

The regression equation represent BC synthesis (Y) was formulated in values at significant terms (P<0.05) as following:

dw/l = -3.42 + 7.570 Ethanol concentration(%) + 1.233 incubation time(day)
- 4.699 Ethanol concentration(%)*Ethanol concentration(%)
- 0.0469 incubation time(day)*incubation time(day) + 0.2000 Ethanol concentration(%) *incubation time(day)

This regression equation has coefficient (R2) was R^2 of 98.80 %, R^2_{pred}= 93.0% and the R^2_{adj} was 98.08 %. The results showed that ethanol concentration and incubation time exerted significant individual and quadratic effects (p < 0.05).

Table 5: Regression analysis of BC production by *K. rhaeticus* N1 MW32270 in CCD

Source	DF	Adjust Sum of squares	Adjust Mean of square	F-Value	P-Value
Model	5	56.4588	11.2918	123.28	0
Linear	2	31.1417	15.5708	169.99	0
Ethanol (%)	1	13.8017	13.8017	150.68	0
Inc. time (days)	1	17.34	17.34	189.31	0
Square	2	24.7547	12.3773	135.13	0
Ethanol (%) × Ethanol (%)	1	19.2947	19.2947	210.65	0
Inc. time (days) × Inc. time	1	0.2373	0.2373	2.59	0.152
2-Way Interaction	1	0.5625	0.5625	6.14	0.042
Ethanol (%) × Inc. time	1	0.5625	0.5625	6.14	0.042
Error	7	0.6412	0.0916		
Lack of fit	3	0.4492	0.1497	3.12	0.15
Pure Error	4	0.192	0.048		
Total	12	57.1			

R^2 = 98.80 %; R^2_{adj} = 98.08 %; R^2_{pred}= 93.0%

The interactions between two variables (ethanol concentration and incubation time) with BC production were presented by response surface plots and contour plots (fig.4). The 3D plot (figure 4a) and their respective 2D contour plots (figure 4b) provide a visual interpretation of the interaction between two factors. In the response surface plot, levels of ethanol concentration and incubation time are varied from (0-1.5% and 5-10day respectively). It can be seen that BC yield increased with an increase in incubation time while in the case of ethanol concentration there is an increase in BC production with increasing ethanol concentration from 0 to 1.0%.

The corresponding Contour plots indicate different regions of yields based on different colors. The height of the surface represents the value of BC production. The contour plots are curved lines because the model contains the interactions of the factors. The maximum yield falls in the range >8 g/l as indicated by the dark shaded region in the plot. Hence to optimize the levels of ethanol concentration and incubation time, it is necessary to carry out experiments in this region of higher yield.

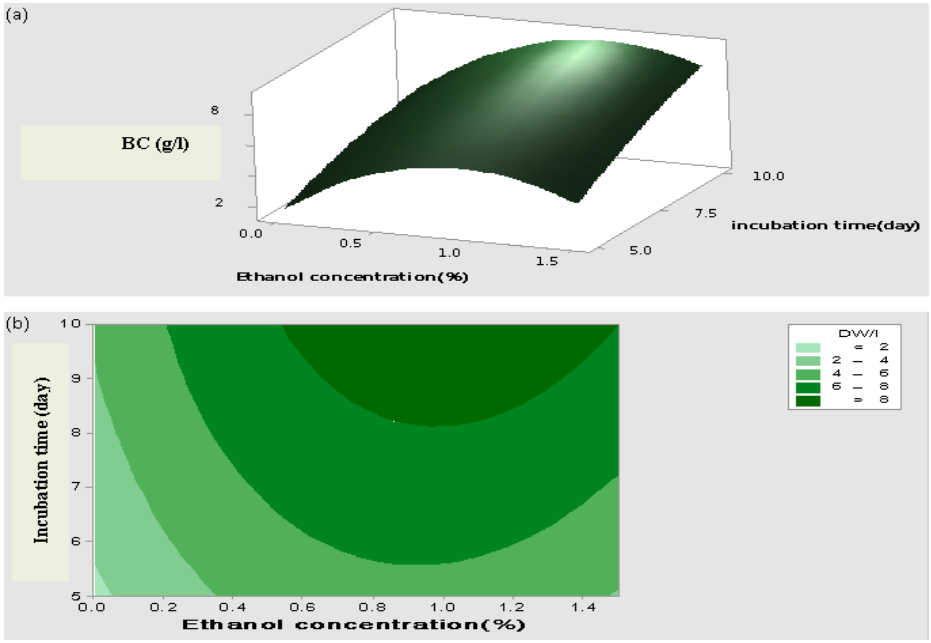

Fig. 4: 3D response surface (a) plots and contour plots (b) for BC production by *K. rhaeticus N1 MW32270* showing the interaction effects of ethanol concentration (%) and incubation time (days)

Validation of BC production by *K. rhaeticus N1 MW32270*

To confirm statistical results, the fitted value of ethanol concentration and incubation time was used in cultural medium, and then BC production was compared with the predicted value obtained from the optimization plot. According to the achieved results, the BC production by *K. rhaeticus N1 MW32270* was 9.2± 1.1g/l in optimized medium, which agreed well with the predicted values 9.1 g/l, indicating that the model was valid. The BC production by *K. rhaeticus N1 MW32270* in optimized medium was then compared with BC production in standard HS medium (Table 6). The results showed that BC production increased by 115% when compared with BC production when original HS medium was used.

Table 6: BC production by *K. rhaeticus N1 MW32270* grown on HS and optimized medium

Bacterial strain	Medium	BC production (g/l)
K. rhaeticus N1 MW32270	HS medium	4.3± 0.5
	Optimized medium	9.2± 1.1

DISCUSSION

To produce a high yield of BC by *K. rhaeticus N1 MW32270*, statistical optimization was done. PBD revealed that among the eight tested parameters, ethanol concentration and incubation time were the most significant factors affecting BC production. CCD was used for the optimization of ethanol concentration and

incubation time. The higher BC yield of 9.2 g/l was obtained after 10 days and 1.015% of ethanol. Several studies reported that ethanol has an impact effect on BC production which stimulates BC production[21-23]. Son *et al.* [24] found that BC production increased about 4 times by adding 1.4% (v/v) ethanol to modified HS medium. Addition of 1% (v/v) ethanol to the broth medium decreased the numbers of non cellulose forming cells of *G. hansenii* strain [21].

Chawla *et al*., [25] reported that the role of ethanol is to degenerate the BC non-forming cells of *G. hansenii.*

It was proved that the greatest increase in the weight of BC takes place after 7-8 days[26-28]. BC was easily visible on surface of the broth medium after 48 hours of incubation. The amount of synthesized BC increased until 8 days[27]. Raghunathan**,** [29] reported that BC production starts after 24 hours of incubation and reaches to a maximum after 10 days; thereafter there was no increase in the BC yield.

Data obtained revealed that incubation temperature at 25°C is more favorable for BC production as shown in fitted mean plots and Coefficient value (-0.200) . Most studies revealed that the optimal growth temperature for BC synthesis ranged from 25 to 30°C[19,24].

The coefficient value for pH was 0.167 indicate that increasing of pH value from 5 to 7 is more favorable for BC production. pH 7.0 supported the maximum yield of BC [29]**.**

In the present study there was no remarkable difference in BC production when glucose concentration was 2% or 4%. Using of glucose at concentration of 2% and 4% was previously reported[24,30]**.** The effect of initial glucose concentration on BC production is important, higher concentrations of glucose lead to formation of gluconic acid (as a by- product) which decreases the pH of the culture medium thus, decreases BC production [25].

The regression equation has coefficient (R2) was R^2 of 98.80 %, implying that the model capability to explain R^2 of 98.80 %, of the variability. The R^2_{pred}= 93.0% is in level-headed concurrence with the R^2_{adj} = 98.08%, because the Predicted R2 and adjusted R2 to be in ‘‘reasonable agreement” must be inside approximately 20% of each other [31]**.**

CONCLUTION

BC is an interesting biomaterial that has been successfully used in many special applications such as wound dressing, blood vessels and scaffold for tissue regeneration. This work has demonstrated the use of PBD and RSM for determining the optimum conditions which are required to obtain optimum yield of BC produced by *K. rhaeticus N1 MW322708* strain. From Pareto chart of standardized effects, it can be seen that ethanol concentration and incubation time have significant effect on BC production when compared to other tested factors. Hence to optimize BC production it is necessary to optimize the concentration of ethanol and incubation time using different concentrations of ethanol and different incubation time, whereas other components of the medium can be kept constant. Our results showed that BC production increased by 115% when compared with BC production in the standard HS medium. Response 3D plot and their respective 2D contour plots are very helpful in visualizing the main effects and interaction of factors. Hence a statistical experimental design maximizes the amount of data that can be obtained, while reduces the numbers of individual experiments required. PBD and RSM are powerful tools for the rapid search of significant factors from many factors, reducing the error and the optimization of factors can achieved in an economical approach.

Ethical statement:

This article does not contain any studies with human participants or animals performed by any of the authors.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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